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Form PTO 1390 (REV 5-93) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED / ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371	ATTORNEY'S DOCKET NUMBER B45122 U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) 09/554860

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP98/07521	16 November 1998	19 November 1997

TITLE OF INVENTION
RECOMBINANT ALLERGEN WITH REDUCED ENZYMATIC ACTIVITY

APPLICANT(S) FOR DO/EO/US
Claudine BRUCK, Alex BOLLEN, Paul JACOBS and Marc MASSAER

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1 [x] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. [x] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. [x] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. [x] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [x] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).

6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. [x] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [x] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.

8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S. C. 371(c)(3)).

9. [x] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. [x] An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.

12. [x] An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.

13. [x] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
    [x] Please amend the specification by inserting before the first line the sentence: This is a 371 of International Application PCT/EP98/07521, filed 16 November 1998, which claims benefit from the following Foreign Application: GB 9724531.0 filed 19 November 1997.

14. [ ] A substitute specification.

15. [ ] A change of power of attorney and/or address letter.

16. [ ] Other items or information:

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US APPLICATION NO. (if known see 37 CFR 1.50) 09/554860	INTERNATIONAL APPLICATION NO. PCT/EP98/07521	ATTORNEYS DOCKET NO. B45122

				CALCULATIONS	PTO USE ONLY
17. [X] The following fees are submitted:					
Basic National Fee (37 C.F.R. 1.492(a)(1)-(5)):					
Search Report has been prepared by the EPO or JPO **$840.00**					
International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) **$670.00**					
No International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) **$690.00**					
Neither International Preliminary Examination Fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO............**$970.00**					
International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)................**$96.00**					
ENTER APPROPRIATE BASIC FEE AMOUNT =				**$840.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				**$0.00**	
Claims	Number Filed	Number Extra	Rate		
Total claims	**21 - 20 =**	**1**	**1 x $18.00**	**$18.00**	
Independent claims	**7 - 3 =**	**4**	**4 x $78.00**	**$312.00**	
Multiple dependent claims (if applicable)			**+ $260.00**	**$260.00**	
TOTAL OF ABOVE CALCULATIONS =				**$590.00**	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				**$**	
SUBTOTAL =				**$1430.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)) +				**$**	
TOTAL NATIONAL FEE =				**$1430.00**	
				Amount to be refunded	$
				charged	$

a. ☐ A check in the amount of $_____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 19-2570 in the amount of **$1430.00** to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-2570. A duplicate copy of this sheet is enclosed.

d. ☒ General Authorization to charge any and all fees under 37 CFR 1.16 or 1.17, including petitions for extension of time relating to this application (37 CFR 1.136 (a)(3)).

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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"EXPRESS MAIL CERTIFICATE" 526 Rec'd PCT/PTO 19 MAY 2000
"EXPRESS MAIL" MAILING LABEL NUMBER EL229502551US
DATE OF DEPOSIT 19 May 2000

Attorney Docket No. B45122

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Bruck, et al.	19 May 2000
Int'l. App. No.:	PCT/EP98/07521	Group Art Unit No.: Unknown
Int'l. Filing Date:	16 November 1998	Examiner: Unknown

For: RECOMBINANT ALLERGEN WITH REDUCED ENZYMATIC ACTIVITY

Assistant Commissioner of Patents
Box: PCT
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Preliminary to the examination of this application, applicants respectfully request amendment of the above-identified application as follows:

IN THE CLAIMS:

Please cancel claim 21.

Please amend claim 18.

18. (Amended) A vaccine comprising a recombinant mutant allergen as claimed in any one of claims 1 to [17] 16, and an adjuvant.

REMARKS

The above-identified application is being entered into the National Phase from PCT application no. PCT/EP98/07521. Applicants have cancelled claim 21 and amended claim 18 to put the claims in conformity with U.S. practice.

Intl. App. No.: PT/EP98/07521
Docket No. B45122

No new matter has been introduced.

Respectfully submitted,

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09/554860
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RECOMBINANT ALLERGEN WITH REDUCED ENZYMATIC ACTIVITY

The present invention relates to novel therapeutic formulations. said formulations being effective in the reduction of allergic responses to specific allergens. Further, this invention relates to novel polynucleotides, polypeptides encoded by them and to the use of such polynucleotides and polypeptides, and to their production. In particular, novel vaccines are provided comprising polypeptides and to their use in the treatment of humans suffering from allergies or prevention of individuals at risk from allergies, preferably said vaccines comprising a recombinant mutant *Dermatophagoides pteronyssinus* allergen Der P1.

Allergic responses in humans are common, and may be triggered by a variety of allergens. Allergic individuals are sensitised to allergens, and are characterised by the presence of high levels of allergen specific IgE in the serum. and possess allergen specific T-cell populations which produce Th2-type cytokines (IL-4, IL-5, and IL-13). Binding of IgE, in the presence of allergen, to Fc receptors present on the surface of mastocytes and basophils. leads to the rapid degranulation of the cells and the subsequent release of histamine, and other preformed and neoformed mediators of the inflammatory reaction. In addition to this, the stimulation of the T-cell recall response results in the production of IL-4 and IL-13, together cooperating to switch B-cell responses further towards allergen specific IgE production. For details of the generation of early and late phase allergic responses see Joost Van Neeven *et al*.. 1996, Immunology Today, 17, 526. In non-allergic individuals. the immune response to the same antigens may additionally include Th1-type cytokines such as IFN-γ. These cytokines may prevent the onset of allergic responses by the inhibition of high levels of Th2-type immune responses, including high levels of allergen specific IgE. Importantly in this respect, is the fact that IgE synthesis may be controlled by an inhibitory feedback mechanism mediated by the binding of IgE/allergen complexes to the CD23 receptor on B-cells (Luo et al., J.Immunol.. 1991, 146(7), 2122-9; Yu et al., 1994, Nature, 369(6483):753-6). In systems that lack cellular bound CD23, this inhibition of IgE synthesis does not occur.

Current strategies in the treatment of such allergic responses include means to prevent the symptomatic effects of histamine release by anti-histamine treatments and/or local administration of anti-inflammatory corticosteroids. Other strategies which are under development include those which use the hosts immune system to prevent the degranulation of the mast cells, Stanworth *et al.*, EP 0 477 231 B1. Other forms of immunotherapy have been described (Hoyne *et al.*, J.Exp.Med., 1993, 178, 1783-1788; Holt *et al.*, Lancet, 1994, 344, 456-458).

Some common allergens present in bee venom, house dust mite emanations and parasite proteins have been found to induce mast cell degranulation, and to stimulate interleukin-4 synthesis and secretion, even in the absence of allergen-specific IgE (Machado et al, 1996, Eur.J.Immunol. 26, 2972-2980). This non-immunological degranulation by proteolytic allergens, such as bee venom phospholipase A2 or proteases associated with house dust mite emanations is dependent on enzymatic activity.

The present invention provides recombinant mutant allergens having significantly reduced proteolytic activity relative to the wild-type proteolytically active allergen, as well as nucleic acids encoding the same, and their use as a prophylactic or immunotherapeutic agent against allergy. A preferred allergen is the house dust mite allergen Der p1.

The present invention relates to the provision of formulations for the treatment and prophylaxis of allergy, by providing means to down-regulate the production of IgE, as well as modifying the cell mediated response to the allergen, through a shift from a Th2 type to a Th1 type of response (as measured by the reduction of ratio of IL-4 : IFN-γ producing DerP1 specific T-cells, or alternatively a reduction of the IL-5:IFN-γ ratio). This is achieved by the provision and use of recombinant mutant allergens with impaired enzymatic activity.

DerP1, a group 1 protease allergen of the house dust mite *Dermatophagoides pteronyssinus* (Topham *et al.*, 1994, Protein Engineering, 7, 7, 869-894; Simpson *et al.*, 1989, Protein Sequences and Data Analyses, 2, 17-21) is one such allergen. It is a 30

KDa protein and has been cloned and sequenced (Chua *et al.*, 1988, J.Exp.Med., 167, 175-182). It is known to contain 222 amino acid residues in the mature protein. The sequence of DerP1 shares 31% homology to Papain, and importantly shares homology in the enzymatically active regions, most notably the Cys34-His170 ion pair (*Topham et al., supra*). DerP1 is produced in the mid-gut of the mite, where its role is probably related to the digestion of food. Up to 0.2 ng or proteolytically active DerP1 is incorporated into each fecal pellet, each around 10-40 μm in diameter and, therefore, easily inspired into the human respiratory tract. Overnight storage of purified DerP1 preparations at room temperature results in almost complete loss of enzymatic activity due to autoproteolytic degradation (Machado *et al.*, 1996, Eur.J.Immunol. 26, 2972-2980).

DerP1 has been found to cleave the low affinity immunoglobulin IgE Fc receptor from the surface of human B lymphocytes (CD23, Hewitt *et al.*, 1995, J.Exp.Med., 182, 1537-1544) and CD25 (Schultz et al., J.Exp.Med, 1998, 187(2):271-5) the alpha subunit of the human T cell interleukin-2 receptor. Cleavage of the receptor from the B cell surface was associated with a parallel increase in soluble CD23 in the culture supernatant. It has been suggested that the loss of cell surface CD23 from IgE-secreting B cells may promote and enhance IgE immune responses by ablating the important inhibitory feedback mechanism that normally limits IgE synthesis (Hewitt *et al.*, 1995, *J.Exp.Med.*, 182, 1537-1544). Furthermore, since soluble CD23 has been shown to promote IgE production, fragments of CD23 released by DerP1 may directly enhance the synthesis of IgE. In addition to the effects of CD23 cleavage, the cleavage of CD25 from the surface of T-cells induces a decrease in proliferation and INF-gamma secretion, which, consequently, may bias the immune response toward a Th2 type response. Recent papers which relate to the DerP1 antigen are Machado *et al. Eur. J. Immunol.* (1996) 26: 2972-2980; Hewitt *et al., J. Exp. Med.* (1995) 182: 1537-1544; and Schulz *et al. Eur. J. Immunol.* (1995) 25: 3191-3194.

Other mutant allergens having reduced proteolytic activity which form part of the present invention may be based upon other group I cyteine proteases, such as Der fl from *Dermatophagoides farinae* (80% homology to DerP1), as well as the groups III allergens (serine proteases) including DerpIII (Stewart *et al.*, 1992, Immunology, 75,

29-35) and DerpIV (Yaseuda *et al.*, 1993, Clin.Exp.Allergy, 23, 384-390); and the group IV allergens (amylases).

The allergens of the present invention are recombinantly produced. Der p1 proteolytic activity can be impaired by introducing mutations into the cDNA or genomic DNA, either at the enzymatically active site, or at the site of cleavage between the propeptide and the mature molecule. Said mutant allergen having the following advantages over the wild-type allergen: 1) increases the Th1-type aspect of the immune responses in comparison to those stimulated by the wild type allergen, thereby leading to the suppression of allergic potential of the vaccinated host, and 2) having reduced allergenicity thus being more suitable for systemic administration of high doses of the immunogen, 3) will induce DerP1 specific IgG which compete with IgE for the binding of native DerP1.

The allergens of the present invention are also more stable than isolated or recombinant active DerP1, as measured by the lack of autoproteolytic degradation. Thus, the present invention also provides allergens which are stable compared to the wild-type form of the allergen, said allergens having significantly reduced proteolytic activity and being substantially full length proteins, optionally said allergens further comprising the pro-form of allergen.

One aspect of the present invention provides a nucleic acid encoding mutated Der p1 as set out above, and a further aspect of the invention provides mutated Der p1 *per se*. A yet further aspect of the present invention provides substantially stable recombinant DerP1. Said stable DerP1 being of substantially full length mature protein, or mature protein further comprising the pro-DerP1 section. The term “stable” in the context of the present invention is a product which does not undergo a substantial amount of decomposition by autoproteolysis when incubated overnight at room temperature in comparison to proteolytically active wild-type DerP1, as evidenced by SDS PAGE analysis.

A still further aspect of the invention provides a process for the preparation of a mutated Der p1 protein, which process comprises expressing DNA encoding the said protein in a recombinant host cell and recovering the product.

A DNA molecule encoding a mutated Der p1 (or other mutated allergen) forms a further aspect of the invention and can be synthesized by standard DNA synthesis techniques, such as by enzymatic ligation as described by D.M. Roberts et al in Biochemistry 1985, 24, 5090-5098, by chemical synthesis, by in vitro enzymatic polymerization, or by a combination of these techniques.

Enzymatic polymerisation of DNA may be carried out in vitro using a DNA polymerase such as DNA polymerase I (Klenow fragment) in an appropriate buffer containing the nucleoside triphosphates dATP, dCTP, dGTP and dTTP as required at a temperature of 10^{o}-37^{o}C, generally in a volume of 50ml or less. Enzymatic ligation of DNA fragments may be carried out using a DNA ligase such as T4 DNA ligase in an appropriate buffer, such as 0.05M Tris (pH 7.4), 0.01M $MgCl_2$, 0.01M dithiothreitol, 1mM spermidine, 1mM ATP and 0.1mg/ml bovine serum albumin, at a temperature of 4^{o}C to ambient, generally in a volume of 50ml or less. The chemical synthesis of the DNA polymer or fragments may be carried out by conventional phosphotriester, phosphite or phosphoramidite chemistry, using solid phase techniques such as those described in 'Chemical and Enzymatic Synthesis of Gene Fragments - A Laboratory Manual' (ed. H.G. Gassen and A. Lang), Verlag Chemie, Weinheim (1982),or in other scientific publications, for example M.J. Gait, H.W.D. Matthes, M. Singh, B.S. Sproat, and R.C. Titmas, Nucleic Acids Research, 1982, 10, 6243; B.S. Sproat and W. Bannwarth, Tetrahedron Letters, 1983, 24, 5771; M.D. Matteucci and M.H Caruthers, Tetrahedron Letters, 1980, 21, 719; M.D. Matteucci and M.H. Caruthers, Journal of the American Chemical Society, 1981, 103, 3185; S.P. Adams et al., Journal of the American Chemical Society,1983, 105, 661; N.D. Sinha, J. Biernat, J. McMannus, and H. Koester, Nucleic Acids Research, 1984, 12, 4539; and H.W.D. Matthes et al., EMBO Journal, 1984, 3, 801.

Alternatively, the coding sequence can be derived from DerP1 mRNA, using known techniques (e.g. reverse transcription of mRNA to generate a complementary cDNA strand), and commercially available cDNA kits.

The invention is not limited to the specifically disclosed sequence, but includes any proteolytic allergen which has been mutated to remove some or all of its proteolytic activity, whilst retaining the ability to stimulate an immune response against the wild-type allergen. The proteolytic activity of the mutant allergens may be compared to the wild type by a CD23 cleavage assay according to Shultz *et al.*, 1995, European Journal of Immunology, 25, 3191-3194), or enzymatic degradation of substrates described in Machado et al., 1996, Eur.J.Immunol., 26, 2972-2980. The immunogenicity of the mutant allergen may be compared to that of the wild-type allergen by various immunologicals assays. The cross-reactivity of the mutant and wild-type allergens may be assayed by *in vitro* T-cell assays after vaccination with either mutant or wild-type allergens. Briefly, splenic T-cells isolated from vaccinated animals may be restimulated *in vitro* with either mutant or wild-type allergen followed by measurement of cytokine production with commercially available ELISA assays, or proliferation of allergen specific T cells may be assayed over time by incorporation of tritiated thymidine. Also the immunogenicity may be determined by ELISA assay, the details of which may be easily determined by the man skilled in the art. Briefly, two types of ELISA assay are envisaged. First, to assess the recognition of the mutant DerP1 by sera of mice immunized with the wild type Der p1; and secondly by recognition of wild type DerP1 allergen by the sera of animals immunised with the mutant allergen. Briefly, each wells will be coated with 100 ng of purified wild type or mutated Der p1 overnight at 4°C. After incubating with a blocking solution (TBS-Tween 0.1% with 1% BSA) successive dilutions of sera will be incubated at 37°C for 1 hour. The wells are washed 5 times, and total IgG revealed by incubating with an anti-IgG antibody conjugated with Alkaline phosphatase.

The reduction of enzymaticaly active allergen or DerP1 may be performed by introducing mutations into the native sequence before recombinantly producing the inactivated mutants. This may be achieved by: introducing substitutions, deletions, or additions into the active sites: by inserting, deleting, or substituting residues in regions

of processing the inactive pro-enzyme into the active mature protein; or by altering the three dimensional structure of the protein such that enzymatic activity is lost, this may be achieved, amongst others, by expressing the protein in fragments, or by deleting cysteine residues involved in disulphide bridge formation, or by deleting or adding residues such that the tertiary structure of the protein is substantially altered. Alternatively, mutations may be generated with the effect of altering the interaction between the Cys and the His residues, at positions 34 and 170 of the mature protein respectively (corresponding to positions 132 and 268 of the pre-pro-protein respectively) in the resultant fully folded recombinant protein.

The invention is illustrated herein, but not limited to, three specific mutations which are are given as examples of proteolytically inactive DerP1. First, the enzymatic activity of DerP1 is abrogated by substituting a Cysteine residue in the active site for an alanine. This substitution occurs at Cys132→Ala132 of the pro-DerP1 protein sequence, and is set out in SEQ ID NO. 1. Second, the DerP1 allergen is recombinantly expressed and retained in its inactive pro-protein form by deletion of four amino acid residues at the linker region between the pro- and mature proteins. This deletion removes amino acid residues NAET from the site 96-99 inclusive, from the Pro-DerP1 protein sequence. This sequence is set out in SEQ ID NO. 2. Third, enzymatic activity of DerP1 is abrogated by substituting a Histidine residue in the active site for an alanine. This substitution occurs at His268→Ala268 of the pro-DerP1 protein sequence, and is set out in SEQ ID NO. 3.

The active sites of each wild-type enzymatic allergen may be determined from the literature, or by reference to homologues. For example, the active sites of DerP1, being a cysteine protease, may be putatively inferred by reference to other known cysteine proteases such as Papain. DerP1 shares essential structural and mechanistic features with other papain-like cysteine proteinases, including cathepsin B. The active site thiolate-imidazolium ion pair comprises the side chains of Cys34 and His170 (Topham et al., 1994, Protein Engineering, 7, 7, 869-894).

Mutated versions of Der p 1 may be prepared by site-directed mutagenesis of the cDNA which codes for the Der p 1 protein by conventional methods such as those described by

G. Winter et al in Nature 1982, 299, 756-758 or by Zoller and Smith 1982; Nucl. Acids Res., 10, 6487-6500, or deletion mutagenesis such as described by Chan and Smith in Nucl. Acids Res., 1984, 12, 2407-2419 or by G. Winter et al in Biochem. Soc. Trans., 1984, 12, 224-225.

The process of the invention may be performed by conventional recombinant techniques such as described in Maniatis et. al., Molecular Cloning - A Laboratory Manual; Cold Spring Harbor, 1982-1989.

In particular, the process may comprise the steps of:

1. Preparing a replicable or integrating expression vector capable, in a host cell, of expressing a DNA polymer comprising a nucleotide sequence that encodes the said mutant Der p1 protein;
2. Altering the enzymatic activity of the resultant protein by one of the following techniques: replacing the cysteine or histidine residues (or other residues interacting with other residues within the active site) from the active site with an alanine residue using site directed mutagenesis; replacement of a cDNA fragment by a pair of oligonucleotides whose sequence differ from the natural one; or alternatively, deleting four residues at the junction between the propeptide and the mature enzyme using site directed mutagenesis
3. Transforming a host cell with the said vector
4. Culturing the transformed host cell under conditions permitting expression of the DNA polymer to produce the protein; and
5. Recovering the protein.

The term 'transforming' is used herein to mean the introduction of foreign DNA into a host cell by transformation, transfection or infection with an appropriate plasmid or viral vector using e.g. conventional techniques as described in Genetic Engineering; Eds. S.M. Kingsman and A.J. Kingsman; Blackwell Scientific Publications; Oxford, England, 1988. The term 'transformed' or 'transformant' will hereafter apply to the resulting host cell containing and expressing the foreign gene of interest.

The expression vector is novel and also forms part of the invention.

The replicable expression vector may be prepared in accordance with the invention, by cleaving a vector compatible with the host cell to provide a linear DNA segment having an intact replicon, and combining said linear segment with one or more DNA molecules which, together with said linear segment encode the desired product, such as the DNA polymer encoding the Der p 1 protein under ligating conditions.

Thus, the DNA polymer may be preformed or formed during the construction of the vector, as desired.

The choice of vector will be determined in part by the host cell, which may be prokaryotic or eukaryotic. Suitable vectors include plasmids, bacteriophages, cosmids and recombinant viruses.

The preparation of the replicable expression vector may be carried out conventionally with appropriate enzymes for restriction, polymerisation and ligation of the DNA, by procedures described in, for example, Maniatis et al cited above.

The recombinant host cell is prepared, in accordance with the invention, by transforming a host cell with a replicable expression vector of the invention under transforming conditions. Suitable transforming conditions are conventional and are described in, for example, Maniatis et al cited above, or "DNA Cloning" Vol. II, D.M. Glover ed., IRL Press Ltd, 1985.

The choice of transforming conditions is determined by the host cell. Thus, a bacterial host such as E. coli may be treated with a solution of $CaCl_2$ (Cohen et al, Proc. Nat. Acad. Sci., 1973, 69, 2110) or with a solution comprising a mixture of RbCl, $MnCl_2$, potassium acetate and glycerol, and then with 3-[N-morpholino]-propane-sulphonic acid, RbCl and glycerol. Mammalian cells in culture may be transformed by calcium co-precipitation of the vector DNA onto the cells, by lipofection, or by electroporation. The invention also extends to a host cell transformed with a replicable expression vector of the invention.

Culturing the transformed host cell under conditions permitting expression of the DNA polymer is carried out conventionally, as described in, for example, Maniatis et al and "DNA Cloning" cited above. Thus, preferably the cell is supplied with nutrient and cultured at a temperature below 45°C.

The product is recovered by conventional methods according to the host cell. Thus, where the host cell is bacterial, such as E. coli it may be lysed physically, chemically or enzymatically and the protein product isolated from the resulting lysate. Where the host cell is mammalian, the product may generally be isolated from the nutrient medium or from cell free extracts. Conventional protein isolation techniques include selective precipitation, absorption chromatography, and affinity chromatography including a monoclonal antibody affinity column.

Alternatively, the expression may be carried out either in insect cells using a suitable vector such as a baculovirus, in transformed drosophila cells, or mammalian CHO cells. The novel protein of the invention may also be expressed in yeast cells as described for the CS protein in EP-A-0 278 941.

The vaccine of the invention comprises an immunoprotective amount of the mutated version of the Der p1 (or other) allergenic protein. The term "immunoprotective" refers to the amount necessary to elicit an immune response against a subsequent challenge such that allergic disease is averted or mitigated. In the vaccine of the invention, an aqueous solution of the protein can be used directly. Alternatively, the protein, with or without prior lyophilization, can be mixed, adsorbed, or covalently linked with any of the various known adjuvants. Preferably, the adjuvant may be a preferential inducer of Th1-type immune responses.

An immune response is generated to an antigen through the interaction of the antigen with the cells of the immune system. The resultant immune response may be broadly distinguished into two extreme categories, being a humoral or cell mediated immune responses (traditionally characterised by antibody and cellular effector mechanisms of protection respectively). These categories of response have been termed Th1-type responses (cell-mediated response), and Th2-type immune responses (humoral

response). In mice Th1-type responses are characterised by the generation of antibodies of the IgG2a subtype, whilst in the human these correspond to IgG1 type antibodies. Th2-type immune responses are characterised by the generation of a broad range of immunoglobulin isotypes including in mice IgE, IgG1, IgA, and IgM.

It can be considered that the driving force behind the development of these two types of immune responses are cytokines, a number of identified protein messengers which serve to help the cells of the immune system and steer the eventual immune response to either a Th1 or Th2 response. Thus Th1-type cytokines induce a cell mediated immune response to the given antigen, whilst Th2-type cytokines induce a humoral immune response to the antigen.

It is important to remember that the distinction of Th1 and Th2-type immune responses is not absolute. In reality an individual will support an immune response which is desribed as being predominantly Th1 or predominantly Th2. However, it is often convenient to consider the families of cytokines in terms of that described in murine CD4 T cell clones by Mosmann and Coffman (*Mosmann, T.R. and Coffman, R.L. (1989) THI and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual Review of Immunology, 7, p145-173*). Traditionally, Th1-type responses are associated with cell mediated effector mechanisms such as cytotoxic lymphocytes (CTL) and can be characterised by the production of the INF-γ and IL-2 cytokines by T-lymphocytes. Other cytokines often directly associated with the induction of Th1-type immune responses are not produced by T-cells, such as IL-12. In contrast, Th2- type responses are associated with humoral mechanisms and the secretion of IL-4, IL-5, IL-6, IL-10 and tumour necrosis factor-β (TNF-β).

It is known that certain vaccine adjuvants are particularly suited to the stimulation of either Th1 or Th2 - type cytokine responses. This weighting of cytokine production translates into the generation of either a predominantly Th1-type ot Th2-type immune responses. Traditionally the best indicators of the Th1:Th2 balance of the immune response after a vaccination or infection includes direct measurement of the production of Th1 or Th2 cytokines by T lymphocytes *in vitro* after restimulation with antigen, and measurement of the IgG1:IgG2a ratio of antigen specific antibody responses.

Thus, a Th1-type adjuvant is one which stimulates isolated T-cell populations to produce high levels of Th1-type cytokines when re-stimulated with antigen *in vitro*, and induces antigen specific immunoglobulin responses associated with Th1-type mechanisms (IgG2a in mice, IgG1 in the human).

Adjuvants include, but are not limited to, aluminium hydroxide, muramyl dipeptide and saponins such as Quil A, 3D-MPL (3-O-deacylated monophosphoryl lipid A), or TDM. As a further exemplary alternative, the protein can be encapsulated within microparticles such as liposomes. Particularly preferred adjuvants which preferentially stimulate Th1-type immune responses are combinations of 3D-MPL and QS21 (EP 0 671 948 B1), oil in water emulsions comprising 3D-MPL and QS21 (WO 95/17210), 3D-MPL formulated with other carriers (EP 0 689 454 B1), or QS21 formulated in cholesterol containing liposomes (WO 96/33739), or immunostimulatory oligonucleotides (WO 96/02555). In yet another exemplary alternative, the protein can be conjugated to a carrier protein which is capable of providing T-cell help to the generation of the anti-allergen immune response, such as tetanus toxoid. Use of Quil A is disclosed by Dalsgaard et al., Acta Vet Scand, 18:349 (1977).

Vaccine preparation is generally described in New Trends and Developments in Vaccines, Voller et al. (eds.), University Park Press, Baltimore, Maryland, 1978. Encapsulation within liposomes is described by Fullerton, US Patent 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by Likhite, US Patent 4,372,945 and Armor et al., US Patent 4,474,757.

The amount of the protein of the present invention present in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccines. Such amount will vary depending upon which specific immunogen is employed and whether or not the vaccine is adjuvanted. Generally, it is expected that each dose will comprise 1-1000 μg of protein, preferably 1-200 μg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of antibody titres and other responses in subjects. Following an initial vaccination, subjects will preferably receive a boost in about 4

weeks, followed by repeated boosts every six months for as long as a risk of allergic responses exists.

The vaccines of the present invention may be administered to adults or infants, however, it is preferable to vaccinate individuals soon after birth before the establishment of substantial Th2-type memory responses.

A further aspect of the invention provides a method of preventing or mitigating an allergic disease in man, which method comprises administering to a subject in need thereof an immunogenically effective amount of a mutated allergen of the invention, or of a vaccine in accordance with the invention.

The examples which follow are illustrative but not limiting of the invention. Restriction enzymes and other reagents were used substantially in accordance with the vendors' instructions.

Example 1 - Expression in *Pichia pastoris:*

Construction of pNIV4811

pNIV4811 is designed to promote the expression of mature Der p1 in fusion with the prepropeptide of *Pichia pastoris* MFα. Plasmid ATCC87307 contains the sequence for mature DerP1. The full Derp1 restriction map is given in figure 7.

Ligate with T4 DNA Ligase:

- *Sph*I-*Xho*I from pPIC9k (INVITROGEN V175-20)
- *Xho*I-*Pst*I oligonucleotides whose sequences follow (n^{o} 97038 and n^{o} 97039)
- *Pst*I-*Xba*I from pNIV4810 (plasmid ATCC87307)
- *Avr*II-*Sph*I from pPIC9k

Sequences of the oligonucleotides:

n^{o} 97038

5'TCGAGAAAAGAGAGGCTGAAGCTACTAACGCCTGCA3'

n^{o} 97039

$^{5'}$GGCGTTAGTAGCTTCAGCCTCTCTTTC$^{3'}$

Results

Pichia Pastoris transfected with pNIV4811 leads to the expression of a protein of 43 kD, comprising uncleaved proMFα-mature Der p1 fusion protein, has been detected in several clones (Figure 1).

Construction of pNIV4817

pNIV4817 is derived from pNIV4811. It is designed to promote the expression of the mature Der p1 in fusion with the prepeptide of *Pichia pastoris* MFα.

Ligate:
- *Bst*EII-*Bam*HI from pNIV4811
- *Bam*HI-*Pst*I oligonucleotides n° 97262 and n°97263 whose sequence follows
- *Pst*I-*Bst*EII from pNIV4811

Sequences of the oligonucleotides

n° 97262

$^{5'}$GATCCAAACGATGAGATTTCCTTCAATTTTTACTGCAGTTTTATTCGC AGC ATCCTCCGCATTAGCTGCTCCAACTAACGCCTGCA$^{3'}$

n° 97263

$^{5'}$GGCGTTAGTTGGAGC**AGC**TAATGCGGAGGATGCTGCGAATAAAACTGCAG TAAAAATTGAAGGAAATCTCATCGTTTG$^{3'}$

Results

Several clones expressed the mature form of Der p1 protein with an apparent molecular weight of 30 kDa, which was secreted into the supernatant (Figure 2).

Construction of pNIV4815

Starting from pNIV4811, the following construction is designed to delete four residues [N-A-E-T (T is the first residue of the mature protein)] at the junction between the propeptide and the mature enzyme.

Ligate : *Bln*I - *BamH*I fragment from pPIC9k (the vector used for expression in *P. pastoris*)

*BamH*I - *Eae*I fragment from pNIV4811

*Eae*I - *EcoR*I fragment generated by RT-PCR with primers No 97142 and 97143. Residues : A_6 to E_{74}.

*EcoR*I - *Pst*I oligonucleotides whose sequence follows (No 97140 and 97141). Residues : F75 to C102 except N96AET99

*Pst*I - *Xba*I fragment from pNIV4810.

Sequence of the oligonucleotides allowing the NAET deletion:

No 97140

5'AATTCAAAAACCGATTTTTGATGAGTGCAGAAGCTTTTGAACACCTA AAACTCAATTCGATTTGAACGCCTGCA3' 75 bases

No 97141

5'GGCGTTCAAATCGAATTGAGTTTTGAGGTGTTCAAAAGCTTCTGCAT CATCAAAAATCGGTTTTTG3' 67 bases

RT-PCR Primers

No 97142

5'CATGAAAATTGTTTTGGCCATCGCC3' 25 bases

*Eae*I

No 97143

5'CGGTTTTTGAATTCATCCAACGAC3' 24 bases

*EcoR*I

<u>Construction of pNIV4819</u>

Starting from pNIV4817, an expression plasmid designed to produce the mature form of Der p1 in *Pichia pastoris*, the following construction.is made to replace the cysteine residue from the active site by an alanine residue (corresponding to the Cys 34 mutation in the mature protein).

Ligate: - *Bpu*1102I-AseI fragment from pNIV4817

- AseI-TfiI synthetic fragment resulting from hybridization of oligonucleotides n° 97121 and n° 97122 whose sequence follows: correspondig to residues I_{104} to E_{142} of the proDerP1 (I_{6-} of mature DerP1 protein)

- *Tfi*I-*Bst*EII fragment from pNIV4810 (ATCC 87307)

- *Bst*EII-*Bpu*1102I fragment from pNIV4817

Sequences of the oligonucleotides

n° 97121

5'TAATGGAAATGCTCCAGCTGAAATCGATTTGCGACAAATGCGAACTGTCA
CTCCCATTCGTATGCAAGGAGGCTGTGGTTCAGCTTGGGCTTTCTCTGGTGT
TGCCGCAACTG3'

Ala 113 bases

n° 97122

5'ATTCAGTTGCGGCAACACCAGAGAAAGCCCAAAGCTGAACCACAGCCTCC
TTGCATACGAATGGGAGTGACAGTTCGCATTTGTCGCAAATCGATTTCAGCT
GGAGCATTTCCAT3'

114 bases

Construction of pNIV4815

Starting from pNIV4811, the following construction is made to delete four residues [N-A-E-T (T is the first residue of the mature protein)] at the junction between the propeptide and the mature enzyme.

Ligate : *Bln*I - *BamH*I fragment from pPIC9k (the vector used for expression in *PPIChia pastoris*)

*BamH*I - *Eae*I fragment from pNIV4811

*Eae*I - *EcoR*I fragment generated by RT-PCR with primers No 97142 and 97143. Residues : A_6 to E_{74}.
*EcoR*I - *Pst*I oligonucleotides whose sequence follows (No 97140 and 97141). Residues : F75 to C102 except N96AET99
*Pst*I - *Xba*I fragment from pNIV4810.

Sequence of the oligonucleotides : allowing the NAET deletion.
No 97140
5'AATTCAAAAACCGATTTTTGATGAGTGCAGAAGCTTTTGAACACCTCA
AAACTCAATTCGATTTGAACGCCTGCA3' 75 bases
No 97141
5'GGCGTTCAAATCGAATTGAGTTTTGAGGTGTTCAAAAGCTTCTGCACT
CATCAAAAATCGGTTTTTG3' 67 bases

RT-PCR Primers
No 97142
5'CATGAAAATTGTTTTGGCCATCGCC3' 25 bases
*Eae*I

No 97143
5'CGGTTTTTGAATTCATCCAACGAC3' 24 bases
*EcoR*I

Example 2 - Expression in mammalian cells

Construction of pNIV4812

pNIV4812, an expression plasmid based on pEE14 (CellTech, Cockett *et al.*, 1990 Biotechnology,vol 8,662-667) designed to produce the mature form of Der p1 in CHO-K1, codes for a pre-Der p1 followed by the mature Der p1 sequence (no pro-protein).

Ligate: - *Hind*III-*Xba*I from pEE14
- *Hind*III-*Pst*I oligonucleotides n°97040 and 97041 whose sequence follows

-*Pst*I-*Xba*I from pNIV4810 (plasmid ATCC 87307)

Sequence of the oligonucleotides

n° 97040

5'AGCTTACCATGAAAATTGTTTTGGCCATCGCCTCATTGTTGGCATTGAGCG CTGTTTATGCTCGTACTAACGCCTGCA3'

n° 97041

5'GGCGTTAGTACGAGCATAAACAGCGCTCAATGCCAACAATGAGGCGATGG CCAAAACAATTTTCATGGTA3'

Results

The expression of a protein of an apparent molecular weight of 30 kDa has been detected in several extracts (Figure 3). No protein has been detected in the culture supernatants (data not shown), which suggests that the protein was not secreted from CHO-K1 cells.

<u>Construction of pNIV4814</u>

Starting from pNIV4812, the following construction is made to replace the cysteine residue from the active site by an alanine residue.

Ligate :
- *Afl*II - *Ase*I fragment from pNIV4812.
- AseI-TfiI oligonucleotides as in pNIV4819 construction (No 97121 and 97122)
- *Tfi*I - *Bst*EII fragment from pNIV4810 (ATCC 87307)
- *Bst*EII - *Afl*II fragment from pNIV4812.

Construction of pNIV4819 and pNIV4814 was made possible, thanks to the discovery that in pNIV4810 the codon encoding isoleucine 6 of the mature protein was ATT

instead of ATC as published. This sequence is responsible for the presence of the *Ase*I restriction site.

Construction of pNIV4816

Starting from pNIV4812, designed to expressed in CHO-K1, pNIV4816 has the same deletion as for pNIV4815. This construct results in the production of recombinant proDerP1 with the deletion of the NAET residues from the junction between the pro and mature protein.

Ligate : *Xba*I - *Afl*II fragment from pEE14
*Afl*II - *Eae*I fragment from pNIV4812
*Eae*I - *EcoR*I fragment generated by RT-PCR using primers No 97142 and 97143
*EcoR*I - *Pst*I oligonucleotides No 97140 and 97141 (same oligonucleotides as used in pNIV4815)
*Pst*I - *Xba*I fragment from pNIV4810.

Example 3 - Expression in Drosophila cells

Construction of pNIV4827

pNIV4827 has been designed to promote the expression and secretion of mature Der p1 from baculovirus infected insect cells.

Ligate : pAcGP67A vector linearized with *Pst*I
*Pst*I fragment from pNIV4810 (ATCC 87307)

The expression of Der p1 from pNIV4827 has been demonstrated by western blot.

Construction of pNIV4828

pNIV4828 has been designed to promote the expression and secretion of ProDer p1 from baculovirus infected insect cells.

Ligate : *Sap*I-*Bam*HI from pAcGP67A (Pharmingen ref. 21220P)
*Bam*H1-*Eco*RI 172 bp synthetic fragment
*Eco*RI-*Bss*SI from pNIV4820
*Bss*SI-*Sap*I from pNIV4827

Sequence of the synthetic fragment :

a) coding oligonucleotide N° 97520

$^{5'}$ GAT CCC CGG CCG TCA TCG ATC AAA ACT TTT GAA GAA TAC AAA AAA GCC TTC AAC AAA AGT TAT GCT ACC TTC GAA GAT GAA GAA GCT GCC CGT AAA AAC TTT TTG GAA TCA GTA AAA TAT GTT CAA TCA AAT GGA GGT GCC ATC AAC CAT TTG TCC GAT TTG TCG TTG GAT G$^{3'}$

172 mer

b) complementary sequence N° 97521

$^{5'}$ AAT TCA TCC AAC GAC AAA TCG GAC AAA TGG TTG ATG GCA CCT CCA TTT GAT TGA ACA TAT TTT ACT GAT TCC AAA AAG TTT TTA CGG GCA GCT TCT TCA TCT TCG AAG GTA GCA TAA CTT TTG TTG AAG GCT TTT TTG TAT TCT TCA AAA GTT TTG ATC GAT GAC GGC CGG G $^{3'}$

172 mer

The expression of ProDer p1 from pNIV4828 has been demonstrated by western blot.

Construction of pNIV4832

This plasmid codes for a Der p1 propeptide followed by the mature Der p1 (ProDer p1) sequence and is designed to be expressed in drosophila cells.

Ligate : - *Asp*718-*Bam*HI fragment from expression vector pDS47/V5-His (INVITROGEN V4115-20)

- *Asp*718-*Spe*I synthetic fragment resulting from hybridization of 98023 and 98024 oligonucleotides
- *Spe*I-*Bgl*II fragment from pNIV4828

Sequences of the oligonucleotides

n° 98023

$^{5'}$ GTA CCC TTA AGA TGC TA $^{3'}$

n° 98024

$^{5'}$ CTA GTA GCA TCT TAA GG $^{3'}$

NB : pNIV4828 is a plasmid designed for the isolation of recombinant baculoviruses expressing the pro-Derp 1 fused to gp67 signal peptide.

Results

Transitory expression of pro-DerP1 in drosophila cells has been detected (data not shown).

Construction of pNIV4840

pNIV4840 differs from pNIV4832 in that the expression vector used is stable and inducible (pMT/V5-His)

Ligate : - *Asp*718-*NotI* fragment from pNIV4832

- *NotI*- *Asp*718 from pMT/V5-His (INVITROGEN V4120-20)

Expression of proDerp 1 in drosophila cells has been shown (Figure 4)

Construction of pNIV4842

pNIV4842 was designed to promote the expression and secretion of ProDer p1 from recombinant drosophila cells. ProDer p1 coding sequence was engineered to impair the cleavage of the propeptide. To achieve this goal, four nucleotide triplets coding for NAET including the cleavage site were deleted.

Ligate : - NotI-EcoRI from pNIV4840
- EcoRI-PstI synthetic fragment resulting from hybridization of oligonucleotides n°98136 and n°98137
- PstI-BstEII from pNIV4840
- BstEII-NotI from pNIV4840

Sequence of the synthetic oligonucleotides

a) Coding sequence

N° 98136

5' AAT TCA AAA ACC GAT TTT TGA TGA GTG CAG AAG CTT TTG AAC ACC TCA AAA CTC AAT TCG ATT TGA ACG CCT GCA 3'

75 mer

Complementary sequence

N° 98137

5' GGC GTT CAA ATC GAA TTG AGT TTT GAG GTG TTC AAA AGC TTC TGC ACT CAT CAA AAA TCG GTT TTT G 3'

67 mer

Results

Detection of Der p1 in fusion with its propeptide has been detected in the supernatants after induction (Figure 5). The sequence of this recombinant mutant DerP1 is given in SEQ ID NO. 4.

<u>Construction of pNIV4843</u>

pNIV4843 has been designed to promote the expression and secretion from recombinant drosophila cells of a ProDer p1 form in which the cysteine residue of the active site has been mutated to an alanine.

Ligate : - NotI-Asp718 from pMT/V5-His
- Asp718-PstI from pNIV4832
- PstI-TfiI from pNIV4819
- TfiI-NotI from pNIV4832

Results

Detection of Der p1 in fusion with its propeptide has been detected in the supernatants after induction (Figure 6). The sequence of this recombinant mutant DerP1 is given in SEQ ID NO. 5.

Example 3, Purification procedure of recombinant ProDer p1 secreted from recombinant drosophila cells

Proteins from the spent culture medium (1 liter) were concentrated at 4°C by overnight ammonium sulfate precipitation to 60% saturation. After centrifugation at 17000g during 30 min., the precipitate was resuspended in 20 ml of 20 mM Tris-HCl pH8.0 and dialyzed against 5 liters of the same buffer. Insoluble proteins were discarded by centrifugation at 20000g during 30 min. The dialysate was loaded onto a Q sepharose XL column (3 x 1.6 cm, Pharmacia) equilibrated in 20 mM Tris-HCl pH8.0. After washing the column with the same buffer, bound proteins were eluted by steps of 100 mM increases of NaCl concentration. ProDer p1 mainly eluted at 200mM NaCl. Enriched ProDer p1 fractions were pooled and loaded onto an hydroxyapatite type 1 column (1 x 1.6 cm, Biorad) conditionned in 5 mM potassium phosphate buffer pH 7.0. Unbound material containing ProDer p1 was concentrated by ultrafiltration using Omega membrane (cut-off : 10kD, Filtron). The concentrate was loaded onto a superdex 75 FPLC column (30 x 1 cm, Pharmacia) in PBS pH 7.3. Eluted ProDer p1 from the gel filtration column was more than 80% pure.

Example 4, Vaccine formulation

Vaccines comprising the mutant DerP1 or allergens may be formulated with many common adjuvants. One prefered adjuvant system is an oil in water emulsion described below :

The oil in water emulsion adjuvant formulations used in the present invention are made comprising following oil in water emulsion component: 5% Squalene, 5% α-tocopherol, 2.0% polyoxyethylene sorbitan monooleate (TWEEN 80). The emulsions are prepared as a 2 fold concentrate. All examples used in the immunological experiments are diluted with the addition of extra components and diluents to give either a 1x concentration (equating to a squalene:QS21 ratio (w/w) of 240:1) or further dilutions thereof.

Briefly, TWEEN 80 is dissolved in phosphate buffered saline (PBS) to give a 2% solution in the PBS. To provide 100 ml of a two fold concentrate emulsion, 5ml of DL alpha tocopherol and 5ml of squalene are vortexed to mix thoroughly. 95ml of PBS/TWEEN solution is added to the oil and mixed thoroughly. The resulting emulsion is then passed through a syringe needle and finally microfluidised by using an M110S Microfluidics machine. The resulting oil droplets have a size of approximately 145-180 nm (expressed as z av. measured by PCS). The other adjuvant/vaccine components (QS21, 3D-MPL and antigen) are added to the emulsion in simple admixture.

The antigen containing vaccines used herein are formulated either with full dose SB62 adjuvant to give a high squalene:QS21 ratio (240:1) or with a lower amount of SB62 to give a low ratio formulation (48:1). Other vaccines may optionally be formulated with the addition of cholesterol to the oil phase of the emulsion.

These vaccines are assayed in groups of Balb/c mice. Briefly, groups of 10 mice are immunised intramuscularly 2 times at 3 weeks interval with 2 μg mutant allergen combined with oil in water emulsion adjuvant. 14 days following the second immunisation the production of cytokines (IL-4, IL5 and IFN-γ) are analysed after *in vitro* restimulation of spleen and lymph nodes cells with allergen. Antibody response to wild-type allergen and the isotypic profile induced are monitored by ELISA at 21 days

post II and 14 days post IV.

ART 34 AMDT

Claims

1. A recombinant substantially full length mutant allergen, wherein said mutant allergen has a reduced enzymatic activity compared to the wild-type allergen.

2. A recombinant mutant allergen as claimed in claim 1, wherein said allergen is a mutant of a type I cysteine protease allergen.

3. A recombinant mutant allergen as claimed in claim 1, wherein said allergen a mutant of DerP1 from *Dermatophagoides pteronyssinus.*

4. A recombinant mutant allergen as claimed in claim 3, wherein said mutant DerP1 comprises an active site mutant.

5. A recombinant mutant allergen as claimed in claim 4, wherein said active site mutant DerP1 comprises a mutation of the Cys 34 residue.

6. A recombinant mutant allergen as claimed in claim 5, wherein said mutation of the Cys 34 residue comprises an alanine substitution.

7. A recombinant mutant allergen as claimed in claim 4, wherein said active site mutant DerP1 comprises a mutation of the His 170 residue.

8. A recombinant mutant allergen as claimed in claim 3, wherein said mutant DerP1 comprises a mutation at the site of cleavage between the propeptide and the mature molecule.

9. A recombinant mutant allergen as claimed in claim 8, wherein said mutation at the site of cleavage between the propeptide and the mature molecule comprises a deletion of the residues NAET.

10. A recombinant mutant allergen as claimed in claim 3, wherein said mutation comprises the deletion or substitution of cysteine residues which are involved in disulphide bridge formation.

11. Stable recombinant proDerP1.

12. A recombinant mutant allergen having the sequence as set out in SEQ ID NO. 1

13. A recombinant mutant allergen having the sequence as set out in SEQ ID NO. 2

14. A recombinant mutant allergen having the sequence as set out in SEQ ID NO. 3

15. A recombinant mutant allergen having the sequence as set out in SEQ ID NO. 4

16. A recombinant mutant allergen having the sequence as set out in SEQ ID NO. 5

AMENDED SHEET

17. An isolated nucleic acid molecule encoding a mutated version of an allergen as claimed in any one of claims 1 to 16.

18. A vaccine comprising a recombinant mutant allergen as claimed in any one of claims 1 to 17, and an adjuvant.

19. A vaccine as claimed in claim 18, wherein the adjuvant is a preferential stimulator of Th1-type immune responses.

20. A vaccine as claimed in claim 18, wherein the adjuvant comprises one or both of QS21 and 3-O-deacylated monophosphoryl lipid A.

21. Use of a recombinant mutant allergen in the manufacture of a medicament for the treatment of allergy.

22. A method of treating or preventing allergic responses comprising administering to an individual suffering from or susceptible to allergy a vaccine as claimed in claim 18.

FIGURE 1: Expression of a 43 kDa protein corresponding to mature Der p1 in fusion with the prepeptide MF-alpha of *Pichia pastoris* (construct pNIV4811) in yeast cells. The culture supernatants from various *Pichia pastoris* clones incubated in the absence or presence of methanol (methanol induction for 1 to 5 days indicated on the x axis) have been analyzed by SDS-PAGE and immunoblot analysis using an anti-Der p1 peptide (117-133) polyclonal antibody.

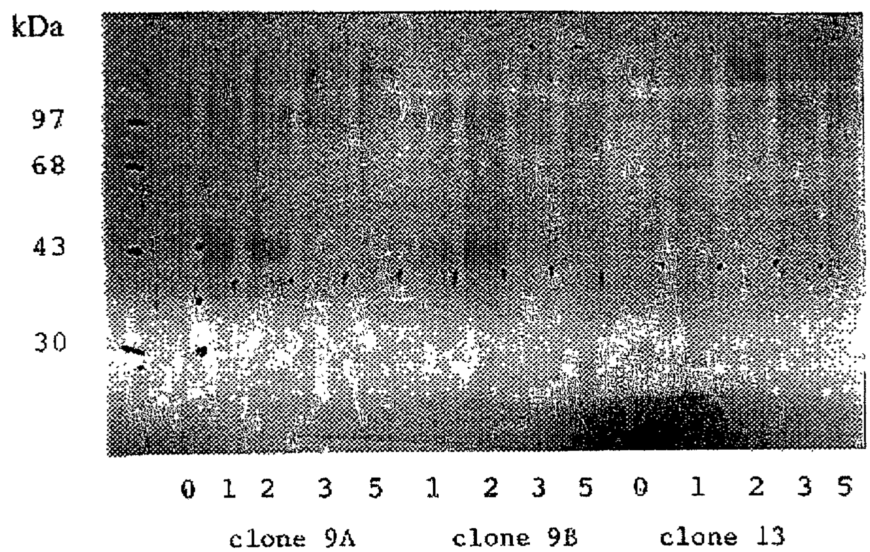

Figure 1

FIGURE 2: Expression of mature Der p1 (30 kDa) in fusion with the prepeptide of *Pichia pastoris* MF-alpha (construct pNIV4817) in yeast cells. The culture supernatants from *Pichia pastoris* cells incubated in the absence (J0) or presence of methanol for 1 day (J1) have been concentrated 50 times and, then, analyzed by SDS-PAGE and immunoblot analysis using an anti-Der p1 peptide (117-133) polyclonal antibody. Arrows indicate the mature Der p1 doublet at about 30 kDa

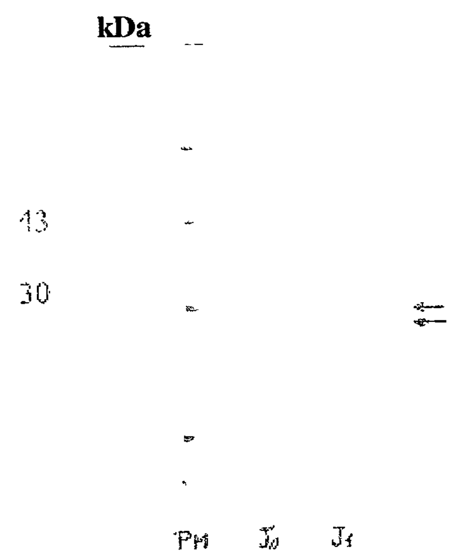

Figure 2

FIGURE 3: Expression of Der p1 in fusion with its propeptide (construct pNIV4812) in CHO-K1 cells. The cell extracts from different clones of CHO-K1 cells transfected with pNIV4812 (lanes 3-8) or transfected with the vector pEE14 alone as negative controls (lane 1 & 2) have been analyzed by SDS-PAGE and immunoblot analysis using an anti-Der p1 peptide (117-133) polyclonal antibody. The arrow indicates the mature Der p1 protein.

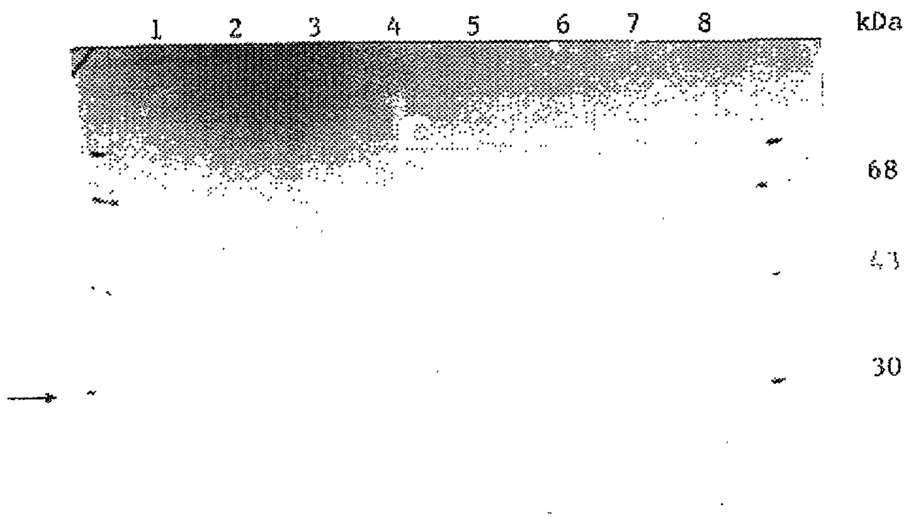

Figure 3

FIGURE 4: Expression of Der p1 in fusion with its propeptide (construct pNIV4840) in drosophila cells S2 (Invitrogen). The cell extracts of different clones of CHO-K1 cells transfected with pNIV4840 (lanes 1 & 4) or transfected with the inducible vector pMT/V5-His alone as negative controls (lanes 2,3,5, & 6) have been analyzed by SDS-PAGE and immunoblot analysis using an anti-Der p1 peptide (117-133) polyclonal antibody. The induction has been carried out for 22 hours (1-3) and 28 hours (4-6).

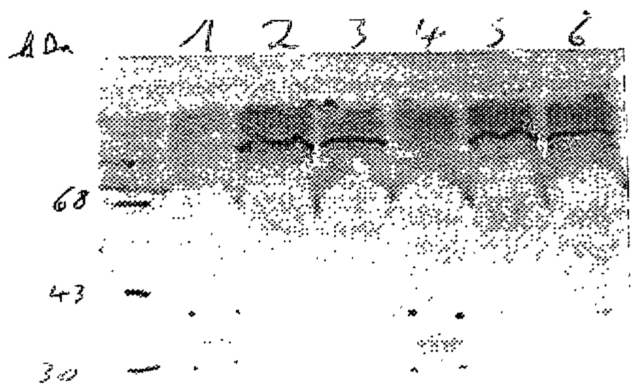

Figure 4

FIGURE 5: Expression of non-cleavable, non-activable Der p1 mutant in fusion with its pro-peptide (construct pNIV4842) in drosophila cells S2 (Invitrogen). The cell supernatants from transiently transfected S2 cells with pNIV4842 (lanes 1-4) or transfected with the inducible vector pMT/V5-His alone as negative control (lanes 5) have been analyzed by SDS-PAGE and immunoblot analysis using an anti-Der p1 peptide (117-133) polyclonal antibody. Lanes 1 to 4 correspond to 1, 4, 5, and 6 days of induction, respectively. Arrows indicate the pro Der p1 doublet at about 36 kDa.

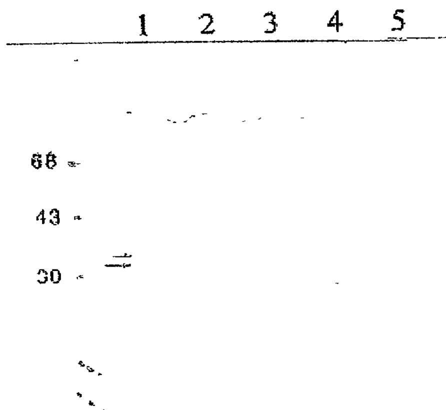

Figure 5

FIGURE 6: Expression of non-active Der p1 mutant in fusion with its propeptide (construct pNIV4843) in drosophila cells S2. The cell supernatants from transiently transfected S2 cells with pNIV4843 (lanes 6-9) or transfected with the inducible vector pMT/V5-His alone as negative control (lanes 5) have been analyzed by SDS-PAGE and immunoblot analysis using an anti-Der p1 peptide (117-133) polyclonal antibody. Lanes 6 to 9 correspond to 1, 4, 5, and 6 days of induction, respectively. Arrows indicate the mature Der p1 doublet at about 36 kDa

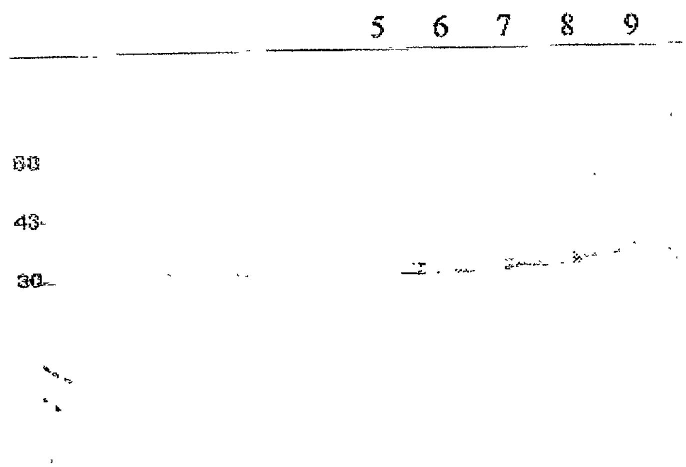

Figure 6

Figure 7, Derp1 restriction map

```
(Linear) MAP of: Derp1.seq  check: 7532  from: 1  to: 963

ID   DP11695    standard; RNA; INV; 1099 BP.

                              BccI
                         CviJI |
                          HaeI |                          HaeII
                        HaeIII |                          HhaI|
                          MscI |                   Eco47III|| CjePI
        Tsp509I           EaeI | |           MnlI    MwoI ||| MwoI|      BccI
              |              | | |              |       | |||    ||         |
     ATGAAAATTGTTTTGGCCATCGCCTCATTGTTGGCATTGAGCGCTGTTTATGCTCGTCCA
   1 ---------+---------+---------+---------+---------+---------+ 60
     TACTTTTAACAAAACCGGTAGCGGAGTAACAACCGTAACTCGCGACAAATACGAGCAGGT

a    M  K  I  V  L  A  I  A  S  L  L  A  L  S  A  V  Y  A  R  P  -

          DpnI
      Sau3AI |                                                 BbvI
       ClaI| |                    CviJI                        NspV|
       TaqI| |          CjePI   MboII |                        TaqI|
          || |              |       | |                          ||
     TCATCGATCAAAACTTTTGAAGAATACAAAAAAGCCTTCAACAAAAGTTATGCTACCTTC
  61 ---------+---------+---------+---------+---------+---------+ 120
     AGTAGCTAGTTTTGAAAACTTCTTATGTTTTTTCGGAAGTTGTTTTCAATACGATGGAAG

a    S  S  I  K  T  F  E  E  Y  K  K  A  F  N  K  S  Y  A  T  F  -

                     Sth132I
                  MboII   |
                 BscGI|   |
             Fnu4HI  ||   |
              AluI|  ||   |
             CviJI|  ||   |
             MboII|  ||   |         HinfI
              TseI|  ||   |          TfiI                    MnlI
                 ||  ||   |             |                       |
     GAAGATGAAGAAGCTGCCCGTAAAAACTTTTTGGAATCAGTAAAATATGTTCAATCAAAT
 121 ---------+---------+---------+---------+---------+---------+ 180
     CTTCTACTTCTTCGACGGGCATTTTTGAAAAACCTTAGTCATTTTATACAAGTTAGTTTA

a    E  D  E  E  A  A  R  K  N  F  L  E  S  V  K  Y  V  Q  S  N  -

          BccI
     Hin4I |
     NgoGV |                             ApoI
     NlaIV |                            EcoRI
     BanI| | MmeI Hpy188IX            Tsp509I     FokI
       | | |    |        |                  |        |
     GGAGGTGCCATCAACCATTTGTCCGATTTGTCGTTGGATGAATTCAAAAACCGATTTTTG
 181 ---------+---------+---------+---------+---------+---------+ 240
     CCTCCACGGTAGTTGGTAAACAGGCTAAACAGCAACCTACTTAAGTTTTTGGCTAAAAAC

a    G  G  A  I  N  H  L  S  D  L  S  L  D  E  F  K  N  R  F  L  -

             AluI               MnlI
            CviJI            Tsp509I
         HindIII |          BcgI   |
        CviRI  | |         BsgI |  |TaqI        BsmI
            |  | |            | |  |   |           |
     ATGAGTGCAGAAGCTTTTGAACACCTCAAAACTCAATTCGATTTGAATGCTGAAACTAAC
 241 ---------+---------+---------+---------+---------+---------+ 300
     TACTCACGTCTTCGAAAACTTGTGGAGTTTTGAGTTAAGCTAAACTTACGACTTTGATTG

a    M  S  A  E  A  F  E  H  L  K  T  Q  F  D  L  N  A  E  T  N  -

          BpmI                                              BsaXI
         PstI|                                              AloI|
       CviRI ||                 AluI                        PpiI|
      Cac8I | ||               CviJI                     MaeIII||
```

```
     SfcI |  ||            MspA1I   ClaI          CjePI   TaaI||
     BcgI| |  ||   CjePI     PvuII   TaqI         MwoI |  Tsp45I||
         || |  ||    |         |       |             | |      |||
         GCCTGCAGTATCAATGGAAATGCTCCAGCTGAAATCGATTTGCGACAAATGCGAACTGTC
     301 ---------+---------+---------+---------+---------+---------+ 360
         CGGACGTCATAGTTACCTTTACGAGGTCGACTTTAGCTAAACGCTGTTTACGCTTGACAG

 a        A  C  S  I  N  G  N  A  P  A  E  I  D  L  R  Q  M  R  T  V  -

                                                           AciI
                 CviRI                                    Fnu4HI
               MnlI |                 NlaIII               TauI
            MslI | |  CviJI DrdII   |CviJI         BsbI   |
               |  | |     |     |     |     |           |   |
         ACTCCCATTCGTATGCAAGGAGGCTGTGGTTCATGTTGGGCTTTCTCTGGTGTTGCCGCA
     361 ---------+---------+---------+---------+---------+---------+ 420
         TGAGGGTAAGCATACGTTCCTCCGACACCAAGTACAACCCGAAAGAGACCACAACGGCGT

 a        T  P  I  R  M  Q  G  G  C  G  S  C  W  A  F  S  G  V  A  A  -

                                                 DpnI
      HinfI  AluI    CviJI                     BstYI |
      TfiI CviJI  MwoI |   TaaI              Sau3AI |  AlwI Tsp509I
          |     |     |  |     |                  | |     |       |
         ACTGAATCAGCTTATTTGGCTTACCGTAATCAATCATTGGATCTTGCTGAACAAGAATTA
     421 ---------+---------+---------+---------+---------+---------+ 480
         TGACTTAGTCGAATAAACCGAATGGCATTAGTTAGTAACCTAGAACGACTTGTTCTTAAT

 a        T  E  S  A  Y  L  A  Y  R  N  Q  S  L  D  L  A  E  Q  E  L  -

                                                  BsaAI
                                                   FokI
                                                   PmlI
                        TaaI   NlaIII            MaeII|
      TaqI          BsbI   |   CjeI |           HphI ||
          |             |    |      | |              |  ||
         GTCGATTGTGCTTCCCAACACGGTTGTCATGGTGATACCATTCCACGTGGTATTGAATAC
     481 ---------+---------+---------+---------+---------+---------+ 540
         CAGCTAACACGAAGGGTTGTGCCAACAGTACCACTATGGTAAGGTGCACCATAACTTATG

 a        V  D  C  A  S  Q  H  G  C  H  G  D  T  I  P  R  G  I  E  Y  -

                                  AluI       MaeII
              MslI              CviJI   ClaI   | BssSI
       CjeI  BstXI|            MmeI |   TaqI   |CviRI|
          |      ||                 | |      |     |   ||
         ATCCAACATAATGGTGTCGTCCAAGAAAGCTACTATCGATACGTTGCACGAGAACAATCA
     541 ---------+---------+---------+---------+---------+---------+ 600
         TAGGTTGTATTACCACAGCAGGTTCTTTCGATGATAGCTATGCAACGTGCTCTTGTTAGT

 a        I  Q  H  N  G  V  V  Q  E  S  Y  Y  R  Y  V  A  R  E  Q  S  -

                           AclI                    ApoI
                          MaeII                   Tsp509I
    NlaIII          CviRI   |                      CjeI|
         |              |   |                          ||
         TGCCGACGACCAAATGCACAACGTTTCGGTATCTCAAACTATTGCCAAATTTACCCACCA
     601 ---------+---------+---------+---------+---------+---------+ 660
         ACGGCTGCTGGTTTACGTGTTGCAAAGCCATAGAGTTTGATAACGGTTTAAATGGGTGGT

 a        C  R  R  P  N  A  Q  R  F  G  I  S  N  Y  C  Q  I  Y  P  P  -

                           AluI
                          CviJI
                         CjeI |
                      HindIII |                  HaeII
                Hpy178III  | ||                  HhaI|
                  ApoI     | ||               Eco47III||
               Tsp509I     | || | CviJI  BcefI      |||   RleAI
                     |     | || |    |      |       |||     |
         AATGTAAACAAAATTCGTGAAGCTTTGGCTCAAACCCACAGCGCTATTGCCGTCATTATT
     661 ---------+---------+---------+---------+---------+---------+ 720
         TTACATTTGTTTTAAGCACTTCGAAACCGAGTTTGGGTGTCGCGATAACGGCAGTAATAA
```

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a      N  V  N  K  I  R  E  A  L  A  Q  T  H  S  A  I  A  V  I  I   -

                                        CviJI
                                       HaeIII
                                         BccI |
                                         EaeI |
                                        GdiII |
           SfaNI    BsmI  HgaI   MslI   | |                  ThaI
               |       |     |      |   | |                     |
       GGCATCAAAGATTTAGACGCATTCCGTCATTATGATGGCCGAACAATCATTCAACGCGAT
   721 ---------+---------+---------+---------+---------+---------+ 780
       CCGTAGTTTCTAAATCTGCGTAAGGCAGTAATACTACCGGCTTGTTAGTAAGTTGCGCTA

a      G  I  K  D  L  D  A  F  R  H  Y  D  G  R  T  I  I  Q  R  D   -

     BstEII                                           MaeIII
     MaeIII                     HincII     MaeIII   TaaI      DraIII
          |                          |          |      |           |
       AATGGTTACCAACCAAACTATCACGCTGTCAACATTGTTGGTTACAGTAACGCACAAGGT
   781 ---------+---------+---------+---------+---------+---------+ 840
       TTACCAATGGTTGGTTTGATAGTGCGACAGTTGTAACAACCAATGTCATTGCGTGTTCCA

a      N  G  Y  Q  P  N  Y  H  A  V  N  I  V  G  Y  S  N  A  Q  G   -

                            CjeI
                           TaaI|
                         BciVI||
                       CjePI|||                                CjeI
                      AlwI |||                               TaaI |
                    RsaI | |||                            CjePI | |
                  SunI | | |||          HgiEII             HphI | |
                 DpnI| | | |||           MunI |            BbvI|| | |
    TaqI   Sau3AI  || | | |||      Tsp509I | |       MaeIII  || | |
       |        |  || | | |||            | |            |  || | |
       GTCGATTATTGGATCGTACGAAACAGTTGGGATACCAATTGGGGTGATAATGGTTACGGT
   841 ---------+---------+---------+---------+---------+---------+ 900
       CAGCTAATAACCTAGCATGCTTTGTCAACCCTATGGTTAACCCCACTATTACCAATGCCA

a      V  D  Y  W  I  V  R  N  S  W  D  T  N  W  G  D  N  G  Y  G   -

                                    Bsp24I
         Fnu4HI     ClaI             CjePI     MboII
           TseI|    TaqI              CjeI|     NdeI|
               ||      |                 ||         ||
       TATTTTGCTGCCAACATCGATTTGATGATGATTGAAGAATATCCATATGTTGTCATTCTC
   901 ---------+---------+---------+---------+---------+---------+ 960
       ATAAAACGACGGTTGTAGCTAAACTACTACTAACTTCTTATAGGTATACAACAGTAAGAG

a      Y  F  A  A  N  I  D  L  M  M  I  E  E  Y  P  Y  V  V  I  L   -

       TAA
   961 --- 963
       ATT
```

SEQ ID NO. 1

Sequence of full mutant DerP1 including pre-protein. Active site mutation Cys 132→Ala 132, corresponding to Cys34→Ala34 of the mature protein). Sequence includes coding and complementary DNA, and amino acid sequences.

```
ATGAAAATTGTTTTGGCCATCGCCTCATTGTTGGCATTGAGCGCTGTTTATGCTCGTCCA 60
---------+---------+---------+---------+---------+---------+
TACTTTTAACAAAACCGGTAGCGGAGTAACAACCGTAACTCGCGACAAATACGAGCAGGT
M  K  I  V  L  A  I  A  S  L  L  A  L  S  A  V  Y  A  R  P   20

TCATCGATCAAAACTTTTGAAGAATACAAAAAAGCCTTCAACAAAAGTTATGCTACCTTC 120
---------+---------+---------+---------+---------+---------+
AGTAGCTAGTTTTGAAAACTTCTTATGTTTTTTCGGAAGTTGTTTTCAATACGATGGAAG
S  S  I  K  T  F  E  E  Y  K  K  A  F  N  K  S  Y  A  T  F   40

GAAGATGAAGAAGCTGCCCGTAAAAACTTTTTGGAATCAGTAAAATATGTTCAATCAAAT 180
---------+---------+---------+---------+---------+---------+
CTTCTACTTCTTCGACGGGCATTTTTGAAAAACCTTAGTCATTTTATACAAGTTAGTTTA
E  D  E  E  A  A  R  K  N  F  L  E  S  V  K  Y  V  Q  S  N   60

GGAGGTGCCATCAACCATTTGTCCGATTTGTCGTTGGATGAATTCAAAAACCGATTTTTG 240
---------+---------+---------+---------+---------+---------+
CCTCCACGGTAGTTGGTAAACAGGCTAAACAGCAACCTACTTAAGTTTTTGGCTAAAAAC
G  G  A  I  N  H  L  S  D  L  S  L  D  E  F  K  N  R  F  L   80

ATGAGTGCAGAAGCTTTTGAACACCTCAAAACTCAATTCGATTTGAATGCTGAAACTAAC 300
---------+---------+---------+---------+---------+---------+
TACTCACGTCTTCGAAAACTTGTGGAGTTTTGAGTTAAGCTAAACTTACGACTTTGATTG
M  S  A  E  A  F  E  H  L  K  T  Q  F  D  L  N  A  E  T  N   100

GCCTGCAGTATCAATGGAAATGCTCCAGCTGAAATCGATTTGCGACAAATGCGAACTGTC 360
---------+---------+---------+---------+---------+---------+
CGGACGTCATAGTTACCTTTACGAGGTCGACTTTAGCTAAACGCTGTTTACGCTTGACAG
A  C  S  I  N  G  N  A  P  A  E  I  D  L  R  Q  M  R  T  V   120

ACTCCCATTCGTATGCAAGGAGGCTGTGGTTCAGCTTGGGCTTTCTCTGGTGTTGCCGCA 420
---------+---------+---------+---------+---------+---------+
TGAGGGTAAGCATACGTTCCTCCGACACCAAGTCGAACCCGAAAGAGACCACAACGGCGT
T  P  I  R  M  Q  G  G  C  G  S  A  W  A  F  S  G  V  A  A   140

ACTGAATCAGCTTATTTGGCTTACCGTAATCAATCATTGGATCTTGCTGAACAAGAATTA 480
---------+---------+---------+---------+---------+---------+
TGACTTAGTCGAATAAACCGAATGGCATTAGTTAGTAACCTAGAACGACTTGTTCTTAAT
T  E  S  A  Y  L  A  Y  R  N  Q  S  L  D  L  A  E  Q  E  L   160

GTCGATTGTGCTTCCCAACACGGTTGTCATGGTGATACCATTCCACGTGGTATTGAATAC 540
---------+---------+---------+---------+---------+---------+
CAGCTAACACGAAGGGTTGTGCCAACAGTACCACTATGGTAAGGTGCACCATAACTTATG
V  D  C  A  S  Q  H  G  C  H  G  D  T  I  P  R  G  I  E  Y   180

ATCCAACATAATGGTGTCGTCCAAGAAAGCTACTATCGATACGTTGCACGAGAACAATCA 600
---------+---------+---------+---------+---------+---------+
TAGGTTGTATTACCACAGCAGGTTCTTTCGATGATAGCTATGCAACGTGCTCTTGTTAGT
I  Q  H  N  G  V  V  Q  E  S  Y  Y  R  Y  V  A  R  E  Q  S   200

TGCCGACGACCAAATGCACAACGTTTCGGTATCTCAAACTATTGCCAAATTTACCCACCA 660
```

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---------+---------+---------+---------+---------+---------+
ACGGCTGCTGGTTTACGTGTTGCAAAGCCATAGAGTTTGATAACGGTTTAAATGGGTGGT
C  R  R  P  N  A  Q  R  F  G  I  S  N  Y  C  Q  I  Y  P  P   220

AATGTAAACAAAATTCGTGAAGCTTTGGCTCAAACCCACAGCGCTATTGCCGTCATTATT 720
---------+---------+---------+---------+---------+---------+
TTACATTTGTTTTAAGCACTTCGAAACCGAGTTTGGGTGTCGCGATAACGGCAGTAATAA
N  V  N  K  I  R  E  A  L  A  Q  T  H  S  A  I  A  V  I  I   240

GGCATCAAAGATTTAGACGCATTCCGTCATTATGATGGCCGAACAATCATTCAACGCGAT 780
---------+---------+---------+---------+---------+---------+
CCGTAGTTTCTAAATCTGCGTAAGGCAGTAATACTACCGGCTTGTTAGTAAGTTGCGCTA
G  I  K  D  L  D  A  F  R  H  Y  D  G  R  T  I  I  Q  R  D   260

AATGGTTACCAACCAAACTATCACGCTGTCAACATTGTTGGTTACAGTAACGCACAAGGT 840
---------+---------+---------+---------+---------+---------+
TTACCAATGGTTGGTTTGATAGTGCGACAGTTGTAACAACCAATGTCATTGCGTGTTCCA
N  G  Y  Q  P  N  Y  H  A  V  N  I  V  G  Y  S  N  A  Q  G   280

GTCGATTATTGGATCGTACGAAACAGTTGGGATACCAATTGGGGTGATAATGGTTACGGT 900
---------+---------+---------+---------+---------+---------+
CAGCTAATAACCTAGCATGCTTTGTCAACCCTATGGTTAACCCCACTATTACCAATGCCA
V  D  Y  W  I  V  R  N  S  W  D  T  N  W  G  D  N  G  Y  G   300

TATTTTGCTGCCAACATCGATTTGATGATGATTGAAGAATATCCATATGTTGTCATTCTC 960
---------+---------+---------+---------+---------+---------+
ATAAAACGACGGTTGTAGCTAAACTACTACTAACTTCTTATAGGTATACAACAGTAAGAG
Y  F  A  A  N  I  D  L  M  M  I  E  E  Y  P  Y  V  V  I  L   320

TAA
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ATT
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SEQ ID NO. 2

Sequence of full mutant DerP1 including pre-protein containing a deletion at the propeptide cleavage site (NAET). Sequence includes coding and complementary DNA, and amino acid sequences.

```
ATGAAAATTGTTTTGGCCATCGCCTCATTGTTGGCATTGAGCGCTGTTTATGCTCGTCCA 60
---------+---------+---------+---------+---------+---------+
TACTTTTAACAAAACCGGTAGCGGAGTAACAACCGTAACTCGCGACAAATACGAGCAGGT
M  K  I  V  L  A  I  A  S  L  L  A  L  S  A  V  Y  A  R  P   20

TCATCGATCAAAACTTTTGAAGAATACAAAAAAGCCTTCAACAAAAGTTATGCTACCTTC 120
---------+---------+---------+---------+---------+---------+
AGTAGCTAGTTTTGAAAACTTCTTATGTTTTTTCGGAAGTTGTTTTCAATACGATGGAAG
S  S  I  K  T  F  E  E  Y  K  K  A  F  N  K  S  Y  A  T  F   40

GAAGATGAAGAAGCTGCCCGTAAAAACTTTTTGGAATCAGTAAAATATGTTCAATCAAAT 180
---------+---------+---------+---------+---------+---------+
CTTCTACTTCTTCGACGGGCATTTTTGAAAAACCTTAGTCATTTTATACAAGTTAGTTTA
E  D  E  E  A  A  R  K  N  F  L  E  S  V  K  Y  V  Q  S  N   60

GGAGGTGCCATCAACCATTTGTCCGATTTGTCGTTGGATGAATTCAAAAACCGATTTTTG 240
---------+---------+---------+---------+---------+---------+
CCTCCACGGTAGTTGGTAAACAGGCTAAACAGCAACCTACTTAAGTTTTTGGCTAAAAAC
G  G  A  I  N  H  L  S  D  L  S  L  D  E  F  K  N  R  F  L   80

ATGAGTGCAGAAGCTTTTGAACACCTCAAAACTCAATTCGATTTG            AAC 300
---------+---------+---------+---------+---------+---------+
TACTCACGTCTTCGAAAACTTGTGGAGTTTTGAGTTAAGCTAAAC            TTG
M  S  A  E  A  F  E  H  L  K  T  Q  F  D  L              N   100

GCCTGCAGTATCAATGGAAATGCTCCAGCTGAAATCGATTTGCGACAAATGCGAACTGTC 360
---------+---------+---------+---------+---------+---------+
CGGACGTCATAGTTACCTTTACGAGGTCGACTTTAGCTAAACGCTGTTTACGCTTGACAG
A  C  S  I  N  G  N  A  P  A  E  I  D  L  R  Q  M  R  T  V   120

ACTCCCATTCGTATGCAAGGAGGCTGTGGTTCATGTTGGGCTTTCTCTGGTGTTGCCGCA 420
---------+---------+---------+---------+---------+---------+
TGAGGGTAAGCATACGTTCCTCCGACACCAAGTACAACCCGAAAGAGACCACAACGGCGT
T  P  I  R  M  Q  G  G  C  G  S  C  W  A  F  S  G  V  A  A   140

ACTGAATCAGCTTATTTGGCTTACCGTAATCAATCATTGGATCTTGCTGAACAAGAATTA 480
---------+---------+---------+---------+---------+---------+
TGACTTAGTCGAATAAACCGAATGGCATTAGTTAGTAACCTAGAACGACTTGTTCTTAAT
T  E  S  A  Y  L  A  Y  R  N  Q  S  L  D  L  A  E  Q  E  L   160

GTCGATTGTGCTTCCCAACACGGTTGTCATGGTGATACCATTCCACGTGGTATTGAATAC 540
---------+---------+---------+---------+---------+---------+
CAGCTAACACGAAGGGTTGTGCCAACAGTACCACTATGGTAAGGTGCACCATAACTTATG
V  D  C  A  S  Q  H  G  C  H  G  D  T  I  P  R  G  I  E  Y   180

ATCCAACATAATGGTGTCGTCCAAGAAAGCTACTATCGATACGTTGCACGAGAACAATCA 600
---------+---------+---------+---------+---------+---------+
TAGGTTGTATTACCACAGCAGGTTCTTTCGATGATAGCTATGCAACGTGCTCTTGTTAGT
I  Q  H  N  G  V  V  Q  E  S  Y  Y  R  Y  V  A  R  E  Q  S   200
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TGCCGACGACCAAATGCACAACGTTTCGGTATCTCAAACTATTGCCAAATTTACCCACCA 660
---------+---------+---------+---------+---------+---------+
ACGGCTGCTGGTTTACGTGTTGCAAAGCCATAGAGTTTGATAACGGTTTAAATGGGTGGT
C  R  R  P  N  A  Q  R  F  G  I  S  N  Y  C  Q  I  Y  P  P   220

AATGTAAACAAAATTCGTGAAGCTTTGGCTCAAACCCACAGCGCTATTGCCGTCATTATT 720
---------+---------+---------+---------+---------+---------+
TTACATTTGTTTTAAGCACTTCGAAACCGAGTTTGGGTGTCGCGATAACGGCAGTAATAA
N  V  N  K  I  R  E  A  L  A  Q  T  H  S  A  I  A  V  I  I   240

GGCATCAAAGATTTAGACGCATTCCGTCATTATGATGGCCGAACAATCATTCAACGCGAT 780
---------+---------+---------+---------+---------+---------+
CCGTAGTTTCTAAATCTGCGTAAGGCAGTAATACTACCGGCTTGTTAGTAAGTTGCGCTA
G  I  K  D  L  D  A  F  R  H  Y  D  G  R  T  I  I  Q  R  D   260

AATGGTTACCAACCAAACTATCACGCTGTCAACATTGTTGGTTACAGTAACGCACAAGGT 840
---------+---------+---------+---------+---------+---------+
TTACCAATGGTTGGTTTGATAGTGCGACAGTTGTAACAACCAATGTCATTGCGTGTTCCA
N  G  Y  Q  P  N  Y  H  A  V  N  I  V  G  Y  S  N  A  Q  G   280

GTCGATTATTGGATCGTACGAAACAGTTGGGATACCAATTGGGGTGATAATGGTTACGGT 900
---------+---------+---------+---------+---------+---------+
CAGCTAATAACCTAGCATGCTTTGTCAACCCTATGGTTAACCCCACTATTACCAATGCCA
V  D  Y  W  I  V  R  N  S  W  D  T  N  W  G  D  N  G  Y  G   300

TATTTTGCTGCCAACATCGATTTGATGATGATTGAAGAATATCCATATGTTGTCATTCTC 960
---------+---------+---------+---------+---------+---------+
ATAAAACGACGGTTGTAGCTAAACTACTACTAACTTCTTATAGGTATACAACAGTAAGAG
Y  F  A  A  N  I  D  L  M  M  I  E  E  Y  P  Y  V  V  I  L   320

TAA
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ATT
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SEQ ID NO. 3

Sequence of full mutant DerP1 including pre-protein. Active site mutation His 268 → Ala 268, corresponding to His170→Ala170 of the mature protein). Sequence includes coding and complementary DNA, and amino acid sequences.

```
ATGAAAATTGTTTTGGCCATCGCCTCATTGTTGGCATTGAGCGCTGTTTATGCTCGTCCA  60
---------+---------+---------+---------+---------+---------+
TACTTTTAACAAAACCGGTAGCGGAGTAACAACCGTAACTCGCGACAAATACGAGCAGGT
M  K  I  V  L  A  I  A  S  L  L  A  L  S  A  V  Y  A  R  P    20

TCATCGATCAAAACTTTTGAAGAATACAAAAAAGCCTTCAACAAAAGTTATGCTACCTTC  120
---------+---------+---------+---------+---------+---------+
AGTAGCTAGTTTTGAAAACTTCTTATGTTTTTTCGGAAGTTGTTTTCAATACGATGGAAG
S  S  I  K  T  F  E  E  Y  K  K  A  F  N  K  S  Y  A  T  F    40

GAAGATGAAGAAGCTGCCCGTAAAAACTTTTTGGAATCAGTAAAATATGTTCAATCAAAT  180
---------+---------+---------+---------+---------+---------+
CTTCTACTTCTTCGACGGGCATTTTTGAAAAACCTTAGTCATTTTATACAAGTTAGTTTA
E  D  E  E  A  A  R  K  N  F  L  E  S  V  K  Y  V  Q  S  N    60

GGAGGTGCCATCAACCATTTGTCCGATTTGTCGTTGGATGAATTCAAAAACCGATTTTTG  240
---------+---------+---------+---------+---------+---------+
CCTCCACGGTAGTTGGTAAACAGGCTAAACAGCAACCTACTTAAGTTTTTGGCTAAAAAC
G  G  A  I  N  H  L  S  D  L  S  L  D  E  F  K  N  R  F  L    80

ATGAGTGCAGAAGCTTTTGAACACCTCAAAACTCAATTCGATTTGAATGCTGAAACTAAC  300
---------+---------+---------+---------+---------+---------+
TACTCACGTCTTCGAAAACTTGTGGAGTTTTGAGTTAAGCTAAACTTACGACTTTGATTG
M  S  A  E  A  F  E  H  L  K  T  Q  F  D  L  N  A  E  T  N    100

GCCTGCAGTATCAATGGAAATGCTCCAGCTGAAATCGATTTGCGACAAATGCGAACTGTC  360
---------+---------+---------+---------+---------+---------+
CGGACGTCATAGTTACCTTTACGAGGTCGACTTTAGCTAAACGCTGTTTACGCTTGACAG
A  C  S  I  N  G  N  A  P  A  E  I  D  L  R  Q  M  R  T  V    120

ACTCCCATTCGTATGCAAGGAGGCTGTGGTTCATGTTGGGCTTTCTCTGGTGTTGCCGCA  420
---------+---------+---------+---------+---------+---------+
TGAGGGTAAGCATACGTTCCTCCGACACCAAGTACAACCCGAAAGAGACCACAACGGCGT
T  P  I  R  M  Q  G  G  C  G  S  C  W  A  F  S  G  V  A  A    140

ACTGAATCAGCTTATTTGGCTTACCGTAATCAATCATTGGATCTTGCTGAACAAGAATTA  480
---------+---------+---------+---------+---------+---------+
TGACTTAGTCGAATAAACCGAATGGCATTAGTTAGTAACCTAGAACGACTTGTTCTTAAT
T  E  S  A  Y  L  A  Y  R  N  Q  S  L  D  L  A  E  Q  E  L    160

GTCGATTGTGCTTCCCAACACGGTTGTCATGGTGATACCATTCCACGTGGTATTGAATAC  540
---------+---------+---------+---------+---------+---------+
CAGCTAACACGAAGGGTTGTGCCAACAGTACCACTATGGTAAGGTGCACCATAACTTATG
V  D  C  A  S  Q  H  G  C  H  G  D  T  I  P  R  G  I  E  Y    180

ATCCAACATAATGGTGTCGTCCAAGAAAGCTACTATCGATACGTTGCACGAGAACAATCA  600
---------+---------+---------+---------+---------+---------+
TAGGTTGTATTACCACAGCAGGTTCTTTCGATGATAGCTATGCAACGTGCTCTTGTTAGT
I  Q  H  N  G  V  V  Q  E  S  Y  Y  R  Y  V  A  R  E  Q  S    200

TGCCGACGACCAAATGCACAACGTTTCGGTATCTCAAACTATTGCCAAATTTACCCACCA  660
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---------+---------+---------+---------+---------+---------+
ACGGCTGCTGGTTTACGTGTTGCAAAGCCATAGAGTTTGATAACGGTTTAAATGGGTGGT
C  R  R  P  N  A  Q  R  F  G  I  S  N  Y  C  Q  I  Y  P  P   220

AATGTAAACAAAATTCGTGAAGCTTTGGCTCAAACCCACAGCGCTATTGCCGTCATTATT 720
---------+---------+---------+---------+---------+---------+
TTACATTTGTTTTAAGCACTTCGAAACCGAGTTTGGGTGTCGCGATAACGGCAGTAATAA
N  V  N  K  I  R  E  A  L  A  Q  T  H  S  A  I  A  V  I  I   240

GGCATCAAAGATTTAGACGCATTCCGTCATTATGATGGCCGAACAATCATTCAACGCGAT 780
---------+---------+---------+---------+---------+---------+
CCGTAGTTTCTAAATCTGCGTAAGGCAGTAATACTACCGGCTTGTTAGTAAGTTGCGCTA
G  I  K  D  L  D  A  F  R  H  Y  D  G  R  T  I  I  Q  R  D   260

AATGGTTACCAACCAAACTATGCTGCTGTCAACATTGTTGGTTACAGTAACGCACAAGGT 840
---------+---------+---------+---------+---------+---------+
TTACCAATGGTTGGTTTGATACGACGACAGTTGTAACAACCAATGTCATTGCGTGTTCCA
N  G  Y  Q  P  N  Y  A  A  V  N  I  V  G  Y  S  N  A  Q  G   280

GTCGATTATTGGATCGTACGAAACAGTTGGGATACCAATTGGGGTGATAATGGTTACGGT 900
---------+---------+---------+---------+---------+---------+
CAGCTAATAACCTAGCATGCTTTGTCAACCCTATGGTTAACCCCACTATTACCAATGCCA
V  D  Y  W  I  V  R  N  S  W  D  T  N  W  G  D  N  G  Y  G   300

TATTTTGCTGCCAACATCGATTTGATGATGATTGAAGAATATCCATATGTTGTCATTCTC 960
---------+---------+---------+---------+---------+---------+
ATAAAACGACGGTTGTAGCTAAACTACTACTAACTTCTTATAGGTATACAACAGTAAGAG
Y  F  A  A  N  I  D  L  M  M  I  E  E  Y  P  Y  V  V  I  L   320

TAA
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ATT
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SEQ ID NO. 4

Amino acid sequence for the mutant DerP1 as encoded by pNIV4842, and shown in figure 5.

1 MLLVNQSHQG FNKEHTSKMV SAIVLYVLLA AAAHSAFAAD PRPSSIKTFE

51 EYKKAFNKSY ATFEDEEAAR KNFLESVKYV QSNGGAINHL SDLSLDEFKN

101 RFLMSAEAFE HLKTQFDLNA CSINGNAPAE IDLRQMRTVT PIRMQGGCGS

151 CWAFSGVAAT ESAYLAYRNQ SLDLAEQELV DCASQHGCHG DTIPRGIEYI

201 QHNGVVQESY YRYVAREQSC RRPNAQRFGI SNYCQIYPPN ANKIREALAQ

251 THSAIAVIIG IKDLDAFRHY DGRTIIQRDN GYQPNYHAVN IVGYSNAQGV

301 DYWIVRNSWD TNWGDNGYGY FAANIDLMMI EEYPYVVIL*

SEQ ID NO. 5

Amino acid sequence for the mutant DerP1 as encoded by pNIV4843, and shown in figure 6.

1 MLLVNQSHQG FNKEHTSKMV SAIVLYVLLA AAAHSAFAAD PRPSSIKTFE

51 EYKKAFNKSY ATFEDEEAAR KNFLESVKYV QSNGGAINHL SDLSLDEFKN

101 RFLMSAEAFE HLKTQFDLNA ETNACSINGN APAEIDLRQM RTVTPIRMQG

151 GCGSAWAFSG VAATESAYLA YRNQSLDLAE QELVDCASQH GCHGDTIPRG

201 IEYIQHNGVV QESYYRYVAR EQSCRRPNAQ RFGISNYCQI YPPNANKIRE

251 ALAQTHSAIA VIIGIKDLDA FRHYDGRTII QRDNGYQPNY HAVNIVGYSN

301 AQGVDYWIVR NSWDTNWGDN GYGYFAANID LMMIEEYPYV VIL*

Docket No.: B45122 PCT/EP98/07521

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

RECOMBINANT ALLERGEN WITH REDUCED ENZYMATIC ACTIVITY

the specification of which (check one)

[ ] is attached hereto.
[X] was filed on 16 November 1998 as Serial No. PCT/EP98/07521
and was amended on (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or Inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Number	Country	Filing Date	Priority Claimed
GB 9724531.0	Great Britain	19 November 1997	Yes

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below.

Application Number	Filing Date

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

Serial No.	Filing Date	Status

I hereby appoint the practitioners associated with the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to that Customer Number:

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Address all correspondence and telephone calls to ZOLTAN KEREKES , SmithKline Beecham Corporation, Corporate Intellectual Proprety-U.S., UW2220, P.O. Box 1539, King of Prussia, Pennsylvania 19406-0939, whose telephone number is 610-270-5024.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00

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